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(54) Title: A METHOD FOR GENERATING ANTIGEN-PRESENTING CELLS

(57) Abstract: Described is a method for the generation of antigen-presenting cells (APC), preferably bone marrow-derived dendritic cells (BMDC) or peripheral blood-derived dendritic cells, as antigen carrier having immunostimulatory properties for anti-infective treatment comprising the steps of (a) pulsing the APC with antigen and (b) treating the APC with a CpG oligonucleotide. Said APC are useful as an immune prophylactic or immune therapeutic agent against diseases like AIDS, tuberculosis, malaria or leishmaniasis.

## **A method for generating antigen-presenting cells**

### **Field of the invention**

The present invention relates to a method for the generation of antigen-presenting cells (APC), preferably bone marrow-derived dendritic cells (BMDC) or peripheral blood-derived dendritic cells (DC), as antigen carrier having immunostimulatory properties for anti-infective treatment and cancer vaccination comprising the steps of (a) exposing the APC to antigen and (b) treating the APC with a CpG oligonucleotide. Said APC are useful as an immune prophylactic or immune therapeutic agent against various cancerous and infectious diseases.

### **Background of the invention**

In the last century, major advances in vaccination and chemotherapy have accounted for the significant success in prevention and control of a variety of infectious diseases. At the beginning of the 21st century, however, infectious diseases remain to be the first cause of morbidity and mortality in developing countries and are still responsible for a significant proportion of public health problems in the developed world. For example, in 1998, the World Health Organization (WHO) reported that AIDS, tuberculosis, malaria and leishmaniasis caused more than 5 million deaths all over the world, and therefore they keep being a major group of human diseases to be targeted in the future.

Interestingly, these four diseases, although they are different in their origin from cancer, have common characteristics when it comes to a comparison of vaccination strategies that have been explored. In general, positive clinical results are missing in cancer vaccination and the various vaccination strategies have not yet yielded into a therapeutic modality of generally broad applicability producing regressions of metastatic lesions in individual patients. It is remarkable that independent of the goal of vaccination against cancer and infections, some features are in common: 1. no reliably effective vaccines are available, 2. chemotherapy is limited, 3. the causing agent is a transformed cell type or an intracellular pathogen, and 4. al-

5        though for some of diseased /infected cells antibodies could be beneficial,  
it is the cellular immune response that is central for mediating protection.  
Therefore, the cellular immune responses have been and still are the fo-  
cus of intense investigations in the last decade, and some of the mecha-  
nisms of induction and maintenance have been revealed. Cellular immu-  
10        nity is mediated by CD4<sup>+</sup> and CD8<sup>+</sup> T cells which recognize proteins after  
they have been processed by APC. Their functions are based on pheno-  
typic features and cytokine profiles. Activation of T cells requires the pres-  
ence of APC, such as B cells and dendritic cells. CD8<sup>+</sup> T cells recognize  
antigens presented in the context of MHC class I molecules and their  
15        activity is mediated through the production of cytokines such as IFN-  
gamma and TNF-alpha, and direct cytolytic mechanisms. On the other  
hand, after recognition of antigens in association with MHC class II  
molecules, CD4<sup>+</sup> T cells become activated and differentiate into functional  
subsets termed Th1 and Th2 cells. Th1 cells typically produce IFN-  
20        gamma, which is the most important mediator for macrophage activation  
and the killing of intracellular microorganisms. The induction of IFN-  
gamma-producing CD4<sup>+</sup> T cells has been shown to be dependent on the  
production of IL-12 by APC after exposure to the pathogen at the initiation  
of the immune response. Thus, in response to many intracellular infections  
25        or other pathologic intracellular alterations related to a cancerous cell, IL-  
12 is the inducer cytokine and IFN-gamma is the effector cytokine. Th2  
cells typically produce IL-4, IL-5, IL-6 and IL-10 which stimulate the  
production of antibodies and are strong inhibitors of the intracellular killing  
by macrophages. A number of reports indicated that there is a striking plasticity in  
30        the ability of a given DC subset to respond to different microbes [2-5],  
suggesting that the type of DC stimulus is a critical factor leading to DC-  
mediated polarization of the Th cell response  
From the experimental murine models of intracellular infections and cancer  
vaccination and the observations of the human counterparts, it is now  
35        widely accepted that the Th1 immune response is protective and espe-  
cially in infectious diseases Th2 is disease-promoting. This was originally  
demonstrated for *Leishmania major* infection in susceptible BALB/c versus  
resistant C57BL/6 mice but it was also later confirmed to hold true for  
other bacterial (mycobacteria, *Salmonella*, *Listeria*), fungal (*Candida*,  
*Cryptococcus*, *Aspergillus*, *Paracoccidioides*) and some viral (HIV) infec-

5        tions. For this reason, the induction of an effective Th1 immune response seems to be a critical requirement for the development of immune prophylactic or immune therapeutic agents against diseases as diverse in origin as cancer and parasite infection.

10       According to the findings described above, a prerequisite for the development of vaccines and therapies against intracellular infection and cancer is the preferential induction of the Th1 arm of cellular immune responses. The currently known factors influencing polarization of CD4<sup>+</sup> T cells include: 1. the local cytokine milieu, 2. the dose and route of antigen administration, 3. the type of APC stimulating the T cell, 4. the "strength" of the  
15       signal, i.e. the affinity of the T cell receptor for the MHC-antigen complex plus timing and density of receptor ligation, and, finally, 5. the presence of immunologically active growth factors. From these factors, the cytokine environment surrounding the newly activated T cell seems to be most im-  
20       portant.

In the last years, laboratories have been particularly interested in the use of the most potent APC, the dendritic cells (DC), as "natural" adjuvants and potent inducers of a Th1 immune response. For this purpose, the  
25       model of murine leishmaniasis was used. It could be shown that after cutaneous infection with *Leishmania major*, only DC are able to migrate and transport the antigen from the skin to the lymph nodes, and are unique in providing the signals for initiation of the primary specific T-cell response. In addition, DC retain parasite antigen in an immunogenic form for prolonged  
30       periods, due to the increased stability of the MHC class II-peptide complexes, and may thus allow the sustained stimulation of parasite-specific T cells that maintain protective immunity against leishmaniasis. These observations prompted the scientists to explore the possibility to use DC as natural adjuvants for immunization against infectious diseases. These  
35       studies demonstrated that members of the DC family, epidermal Langerhans cells (LC), after ex vivo pulsing with *L. major* lysate, can induce long-lasting protection of otherwise susceptible BALB/c mice against subsequent challenges with virulent parasites. This protection was paralleled by a pronounced shift towards a Th1-like pattern, in contrast to the control animals in which a typical Th2 immune response was observed. Thus, one

- 5 can expect that DC can serve as an effective antigen delivery system for vaccination against infectious diseases and open the possibility for a potential use in therapy. This notion is supported by similar results obtained with other models of bacterial, parasitic, fungal and viral infections.
- 10 Availability of DC in sufficient numbers as needed in a therapeutic approach and quality to support clinical treatment of patients will in a long run decide about whether the innate therapeutic potential of DC can be used or not. If this problem is not solved the practical implications will be that DC will stay what they currently are, a powerful research tool.
- 15 DC constitute a rare but heterogeneous population phenotypically distinct from macrophages (DC are CD14<sup>+</sup>). DC are defined by their potency as APC and are distinct from other well known, but less potent, APC such as B cells and macrophages. DC have been shown to be derived from numerous lineages and dynamically shift their phenotype in response to the
- 20 local inflammatory environment. The most powerful DC currently known and desirable for use in vaccination approaches are skin-derived DC sometimes referred to as "Langerhans cells" (LC). However, they constitute only 1-3% of the epidermal cells and their isolation from the skin is complicated. Blood DC represent a similarly small population as they contribute to less than 0.3% of the entire circulating blood-leukocyte population. The lack of adequate culture methods for DC is an additional limitation. Thus, in humans, sizable numbers of naturally occurring LC/DC are not accessible.
- 25 Other reported sources for DC are less differentiated cells like CD34<sup>+</sup> progenitors derived from blood monocytes preparations or the uncommitted bone marrow-derived CD34<sup>+</sup> cells. However, these cells have to be differentiated first ex vivo to acquire a DC phenotype.
- 30 Human monocyte-derived DC currently represents the easiest accessible source of DC. The number of monocytes available from blood is reasonable and the procedures involved are not too inconvenient for the donor. Generally, DC that have been used in vaccine protocols have been generated from monocytes preparations stimulated with IL-4 and GM-CSF or from monocyte derived precursors (CD34<sup>+</sup> cells). In humans, monocytes derived cells incubated without IL-4 become activated macrophages. In the murine system, the use of IL-4 is not required to generate DC. In a first
- 35

5 step of preparation, peripheral blood mononuclear cells (PBMC) are isolated via density centrifugation. By this way, all red blood cells and the granulocytes are lost in one isolation step. Then the PBMC are cultured for 6 days in the presence of GM-CSF and IL-4. At day 6 the cells have lost CD14 (a monocyte lineage marker) and gained CD1a. Classical stimuli like  
10 lipopolysaccharide (LPS) can stimulate the (immature) DC to produce factors like IL-6, IL-8 and IL-12 (p40 and p70).  
Despite of limitations posed by the inaccessibility of cutaneous DC they are the best-studied DC type. Much attention has been given to situations in which CD4+ and CD8+ T lymphocytes play a critical role and need to be  
15 activated. Cutaneous DC present tumor antigen as well as antigens from infectious agents in the context of class I molecules. Furthermore, LC are able to present exogenous antigens loaded onto class I molecules, a function unique to LC/DC and known as cross-priming. Thus DC can stimulate both T cells and B cells. This finding is of great importance because both  
20 the CD4+ and CD8+ T cells are a requirement for protective cancer immunity and need to be activated through class I-presented antigen. In addition to their unique antigen presentation function, cutaneous DC are equipped with extraordinary accessory functions: Together, these exquisite features enable DC to induce primary and secondary immune responses. For this  
25 reason, DC are often referred to as "nature's adjuvant" and this opens attractive options in the therapy of cancer and infectious diseases.  
In immunotherapy of cancer, the role of cancer-specific CD4+ and CD8+ T cells for generating an antigen-specific and therapeutic immune response is undisputed and a prerequisite for concepts that foresee successful vaccination and are focused to prevention and control of cancer. Nevertheless, due to the lack of immunogenic tumor antigens, the absence of accessory signals and/or active immunosuppression, the natural or vaccination-induced immune response often fails and does not help to combat cancer. Experimental work generated from several laboratories indicates  
30 that cutaneous DC present tumor antigens in the context of class I molecules, which is a requirement for the activation of both CD4+ and CD8+ T cells to perform protective cancer immunity.  
35 Dendritic cells (DCs) derived from monocytes have been used by few institutions in their current experimental immunotherapy protocols. The results of the studies are difficult to compare since the DC involved have not been

5 generated following a generally accepted standard and their phenotypes are different. The administration of the DC loaded with tumor-associated proteins or peptides resulted in the induction of immune responses against different types of malignant cells. Clinical responses such as stability of disease and tumor regressions have been reported in some patients, particularly with melanoma, myeloma, follicular non-Hodgkin's lymphoma and prostate cancer.

10 In the clinical trials with DC-based vaccines, a number of important limiting issues have become apparent. These include the optimal source and phenotype of DC, the type of antigen and method of loading DC with antigen, whether to induce differentiation/maturation of DC, the route and timing of immunization, and the appropriate clinical scenario.

The monocyte-derived DC currently used for cancer immunotherapy are not generated following a general standardized scheme. To further explore DC-based approaches, it is therefore very important to establish a protocol for the generation of DC in sufficient amounts and with potent immunostimulatory properties that are similar to those reported for LC (MHC class I-mediated antigen presentation, accessory functions, ability to induce a T helper response). The use of bone marrow precursor cells seems to be an alternative way to generate larger numbers of DC. However, the same questions arise: what stimuli/culture conditions are required to differentiate them to become the ideal antigen carrier?

25 With respect to shifting and modulating immune responses certain products of bacteria and helminthes stimulate APC and as well DC to prime and activate preferentially Th1 or Th2 cells, respectively. Among the preferred bacterial products are the oligonucleotides that have been shown to be immunostimulators of B cells, NK cells, peripheral blood mononuclear cells (PBMC) and blood dendritic cells (see US6429199, US 6207646). In various studies it has been shown by Krieg et al. that an unmethylated cytosine-guanine (CpG) di-nucleotide motif is central for the immunostimulatory property and represented by the general formula:

35 5' X<sub>1</sub> CG X<sub>2</sub> 3'; wherein X<sub>1</sub> is selected from the group consisting of A, G and T; and X<sub>2</sub> is C or T. CpG containing nucleotides have been reported to be in range of 8 to 40 base pairs. However, nucleic acids of any size are immunostimulatory if sufficient immunostimulatory motifs are present

5       The authors (Krieg et al.) demonstrate that CpG activates PBMC and that  
within the various cells types present in monocyte preparations the CpG  
DNA directly activates the macrophages, which respond with a release of  
various cytokines (IL-6, GM-CSF and TNF-alpha). Both B cells and NK  
10       cells have been shown to be specifically activated by ODN 1668. Krieg et  
al. furthermore demonstrate that in contrast to monocyte derived dendritic  
cells it is only the low numbered (0.2%), naturally occurring blood dendritic  
cell that is susceptible to CpG stimulation. Krieg et al. about write *mono-*  
*cyte- derived dendritic cells* in US6429199: "DC can be obtained in large  
15       numbers, ....however upon withdrawal of IL-4 lose their DC character-  
istics, .....IL-4 induces Th2 immune response which may not be optimal a  
specific cytotoxic T cell response; .....We found that monocyte- derived  
dendritic cells are sensitive to LPS but surprisingly are not activated by  
20       CpG motifs. It is believed that the inability of monocyte-derived DC to re-  
spond to CpG might be due to the unphysiologic methods by which these  
cells are prepared." Throughout their work Krieg et al. have profiled the  
natural occurring DC of the peripheral blood as the prime target of CpG  
action. However as it has been pointed out before, these physiological DC  
are because of their limited number not a preferred cell type when it  
comes to large scale use of therapeutic cells in mammals.

25       Thus, the technical problem underlying the present invention is to provide  
in a large scale APC, preferably DC, which can serve as antigen carriers,  
or natural adjuvant, for anti-cancer and anti-infective treatments.

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**Summary of the invention**

The solution to the above technical problem has been achieved by providing the embodiments characterized by the claims and as follows: It could be demonstrated that particular DC, i.e., BMDC or monocyte-derived DC,  
10 can be manipulated in vitro with specific maturation stimuli, i.e., CpG oligonucleotides, resulting in the generation of activated BMDC that exhibit a striking capacity to induce a T cell immune response and protect mammals against an otherwise lethal infection with intracellular pathogens and from cancer.

15 DC were generated from bone marrow progenitors as described by Lutz et al. and the resulting cell population had a typical DC morphology with a myeloid DC phenotype (MHC class II+, CD80+, CD86+, CD40+, ICAM-1+, CD11c+), and potent MHC class I dependent antigen-presenting functions in allogeneic MLR and in a proliferation assay with Leishmania-specific T  
20 hybridoma cells. After 10 days of BMDC culture, the non-adherent cells were collected, resuspended in culture medium containing GM-CSF and pulsed with antigen.

25 As a model system, experimental leishmaniasis with Leishmania major was used. A single vaccination of mice for example with DC which had been pulsed in vitro with Leishmania antigen and treated with a CpG oligonucleotide for maturation (DC/CpG/LeishAg) mediated complete protection against subsequent infection with the parasite Leishmania. Control  
30 mice which obtained Leishmania antigen or the CpG oligonucleotide alone were not protected. Analysis of the underlying immunological mechanism revealed that vaccination with DC/CpG/LeishAg induced a cell-mediated immune response of the protective type, i.e., an immune response mediated by CD4+ type 1 T helper cells (Th1). The protective effect was stable and long-lasting, i.e., more than 20 weeks after secondary challenge the  
35 mice did not exhibit any signs of disease. Using this approach for vaccination against infection or cancer, it can be expected that DC generated from humans or other animals will induce a protective immune response in the treated individual.

5      **Brief description of the drawings**

Figure 1: Lesion development in BALB/c mice vaccinated with BMDC preparations and infected one week later with *L. major* parasites

10      BMDCs were produced and incubated with the different treatments as described in Material and Methods. A and B represent two independent experiments and show the average in footpad swelling for every group  $\pm$  SEM (n=5).

15      **Figure 2: Clinical cure of murine cutaneous leishmaniasis induced by CpG-matured lysate-pulsed BMDC is associated with a significant reduction in parasite burden**

20      Control non-vaccinated mice and protected mice from experiment shown in Fig. 1B were sacrificed and the amount of viable parasites was determined by a limiting dilution procedure (A). Samples from footpad suspensions were smeared, stained with Giemsa and observed with light microscopy (B). Pictures displayed are representative from each group.

25      Figure 3: The pattern of cytokine expression by lymph node cells from protected mice indicates a shift towards a Th1-like immune response

30      Pooled lymph node cell suspensions were prepared from some relevant groups shown in Fig. 1B and were incubated for 72 hours in the absence (open) or presence (filled) of *Leishmania* antigen. Supernatants were assayed for the production of IL-2 (A), IFN-gamma (B) and IL-4 (C) by ELISA.

35      Figure 4: The production of *Leishmania*-specific IgG antibodies in protected mice correlates with a Th1 immune response

Sera from individual mice belonging to the experimental groups shown in Fig. 3 were analyzed for the presence of total IgG (A), IgG1 (B) and IgG2a (C) anti-*Leishmania* antibodies by ELISA. Results are shown as O.D. and

5 the average is indicated with the bar. The ratio of IgG2a/IgG1 was calculated for each mouse and is shown in D.

10 Figure 5: Protection against murine cutaneous leishmaniasis by CpG-matured lysate-pulsed BMDC can be also shown in resistant C57BL/6 mice

BMDC were treated and i.v. injected into mice one week before parasite challenge as described in material and methods. Footpad swelling was then weekly registered (A) and the parasitic load in pooled footpads was qualified after 6 weeks of infection(B).

15 Figure 6: (A) Treatment with CpG-matured lysate-pulsed BMDC mediates solid protection against re-infection

20 Cured mice from the experiment shown in Fig. 1A were re-challenged with  $5 \times 10^5$  infective parasites and the lesion development was followed up weekly.

(B) Evaluation of the therapeutic potential of CpG-matured lysate-pulsed BMDC

25 Mice were infected, i.v. injected with CpG-matured lysate-pulsed BMDC at the time-points indicated in the top of the figure and footpad swelling monitored.

Figure 7: IL-12 expression by BMDC used for vaccination

30 BMDC were generated, treated as indicated for 36 hrs and supernatants were separated from cells by centrifugation. Cells were used to amplify the mRNA for IL-12 p40 and IL-12 p35 subunits by RT-PCR, as described in Material and Methods (A). Supernatants were assayed for IL-12 p70 expression by ELISA (B).

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**Detailed description of the invention**

In one aspect, the present invention relates to a method for the generation of an APC as antigen carrier having immunostimulatory properties for anti-infective and anti-cancer treatment comprising the following steps:

10

(a) exposing the APC to antigen (e.g. by pulsing APC with antigen);

and

(b) treating the APC with a CpG oligonucleotide.

15

APC suitable for the method of the present invention comprise different subsets of the DC family with BMDC or peripheral blood-derived DC being preferred. Methods for the generation of DC and the separation of said cells from non-APC are known to the person skilled in the art and described, e.g., in Lutz et al., J. Immunol. Meth. 223: 77-92 (1999); Romani et al., J. Immunol. Meth. 196: 137-151 (1999); Thurner et al., J. Immunol. Meth. 223: 1-15 (1999). Methods for pulsing of the APC in general or specific DC with the antigen are also known to the person skilled in the art and described, e.g., in Flohé et al., Eur. J. Immunol. 28: 3800-3811 (1998) as well as in Example 1(D), below.

20

The person skilled in the art knows how to carry out treatment of the APC with a CpG oligonucleotide, e.g., by following the instructions given in Example 1(D), below. Steps (a) and (b) can be carried out separately or simultaneously.

25

Preparation of the CpG oligonucleotide can be carried out according to conventional methods (cf. Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, NY, USA).

30

The term "having immunostimulatory properties" comprises the capability of the matured APC to provide a protective immune response.

35

Infectious disease related antigens according to this invention is whole cell lysate and antigen mixtures derived from mycobacteria, chlamydia, influenza virus, HPV, HBV, HCV, EBV origin, and molecular defined antigens such as LeIF, elongation factor 4 and LACK from Leishmania, listeriolysin from Listeria monocytogenes and Toxoplasma gondii antigens such as for instance SAG1 and SAG2.

Human cancer antigens recognized by CD8+ t cells are selected from the group of cancer-testis antigens (e.g. MAGE-3, BAGE, GAGE, NY-ESO-1),

5 melanocyte differentiation antigens (e.g. Melan-A/Mart-1, tyrosinase, gp100), overexpressed antigens (e.g. Her2/neu, erbB1, p53, MUC-1) and point mutated antigens (e.g. beta-Catenin, MUM-1, CDK-4, p53, ras).

10 In a preferred embodiment of the present invention a not naturally occurring dendritic cell (DC) having specific antigen presentation properties in a mammal comprising a specific disease related antigen and a CpG molecule is generated and used. Said DC derives from CD34+ bone marrow cells precursor cells or peripheral blood monocytes and the APC are BMDC or peripheral blood-monocyte derived DC as antigen carrier having  
15 immunostimulatory properties for anti-infective and cancer treatment treatment and the method comprises the following steps:

- (a) obtaining bone marrow cells from femurs and/or tibiae or isolating DC precursor cell from peripheral blood monocyte preparations;
- 20 (b) culturing the cells under conditions allowing to generate DC;
- (c) pulsing the isolated DC with antigen; and
- (d) treating the DC with a CpG oligonucleotide.

25

Methods of the steps (a) and (b) are commonly known and, moreover, described in Example 1(D), below. Steps (c) and (d) are preferably performed simultaneously. If performed sequentially, step (d) is performed before (c).

30

In a more preferred embodiment of the method of the present invention, the CpG oligonucleotide comprises the nucleic acid sequence 5'-TTCATGACGTTCTGATGCT-3'. However nucleic acids represented by the general formula(5' X1 CG X2 3') may be used, wherein X1 is selected  
35 from the group consisting of A, G and T; and X2 is C or T as well. A reasonable length for CpG containing nucleotides has been reported to be in range of 8 to 40 base pairs.

The immunogenicity of the antigens used in this invention may be substantially increased by including adjuvants. A preferred embodiment of a vaccine based on the present invention therefore contains QS21, incomplete

5 Freund's adjuvants, IL-2, IL-12, GM-CSF, MPL or an AGP such as RC-529.

In a further more preferred embodiment of the method of the present invention the APC, preferably BMDC, are characterized by their ability to induce a T-helper immune response. This ability can be assayed by standard assays, e.g., the assay described in Example 5, below.

10

The term "antigen" as used herein comprises a lysate of a pathogenic microorganism, e.g., parasite, (see, for preparation, e.g. Example 1(D), below) or one or more purified proteins of the pathogenic organism. Preferably, the antigen is an isolated protein, or a mixture of such proteins of a microorganism and/or the microorganism is an intracellular pathogen. It is especially preferred that the microbial antigen is selected from the group consisting of (1) cells or an extract, (2) an isolated microbial antigen, (3) an isolated nuclei acid representing the antigen operable linked to a promoter for expressing the isolated antigen, or functional variant thereof, (4) a host cell expressing the isolated polypeptide or a functional variant thereof.

15

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The method of the present invention is useful for providing immune protection against a variety of microorganism, preferably intracellular pathogens (parasites), e.g., HIV, Mycobacterium tuberculosis, Plasmodium, Leishmania, Salmonella, Listeria, Toxoplasma and Chlamydia.

25

The present invention also relates to APC having immunostimulatory properties, preferably BMDC or peripheral blood-derived DC, which are obtainable by the methods of the present invention described above and exemplified in the Examples, below, as well as a pharmaceutical composition containing said cells, preferably in combination with suitable pharmaceutical carriers. Examples of suitable pharmaceutical carriers are well known in the art and comprise buffered aqueous solutions. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Administration of the suitable compositions for vaccination may be effected by different ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular or intradermal administration. The route of administration, of course, depends on the nature of the disease, e.g. kind of pathogen or parasite, and the kind of APC contained in

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5 the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular APC to be administered, time and route of administration, the kind of  
10 pathogen, general health and other drugs being administered concurrently. DC pulsed with antigen and treated with CpG and or pharmaceutical compositions of the present invention may be used as a vaccine. Accordingly, in a further aspect, the present invention relates to a method for inducing an immunological response in a mammal that comprises inoculating the  
15 mammal with DC pulsed with antigen and treated with CpG and or pharmaceutical compositions of the present invention, adequate to produce antibody and/or T cell immune response, including, for example, cytokine-producing T cells or cytotoxic T cells, to protect said animal from disease, whether that disease is already established within the individual or not. An  
20 immunological response in a mammal may also be induced by a method comprises delivering the antigen of the present invention via a vector directing expression of the polynucleotide and coding for the polypeptide in vivo in order to induce such an immunological response to produce cytotoxic and memory T cell or antibody to protect said animal from diseases  
25 of the invention. One way of administering the vector is by accelerating it into the desired cells as a coating on particles or otherwise. Such nucleic acid vector may comprise DNA, RNA, a modified nucleic acid, or a DNA/RNA hybrid. For use as a vaccine, the DC pulsed with antigen and treated with CpG are normally provided as a vaccine formulation (composition). The formulation may further comprise a suitable carrier. A preferred route for administration is parenterally (for instance, subcutaneous, intra-muscular, intravenous, or intra-dermal injection). Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions that may contain anti-oxidants, buffers, bacteriostatics and solutes that render the formulation isotonic with the blood of the  
30 recipient; and aqueous and non-aqueous sterile suspensions that may include suspending agents or thickening agents. The packages formulations may be presented in unit-dose or multi-dose /containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior

5 to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. Another way to enhance immunity may require including cytokines and growth factors such as IL-2, IL-4, IL-12, alpha-IFN, GMC-CSF. The dosage will depend on the specific activity of the vaccine and body weight of the recipient and can be readily determined by routine experimentation.

10 Finally, the present invention relates to use of a APC as described above, preferably a BMDC or peripheral blood-derived DC for the preparation of a pharmaceutical composition, preferably an immune prophylactic composition or immune therapeutic composition, for the treatment of a disease  
15 caused by an intracellular pathogen. Preferred diseases are AIDS, tuberculosis, malaria, salmonellosis, listeriosis, toxoplasmosis or leishmaniasis.

The following examples explain the invention in more detail.

20

#### **Example 1: General methods**

(A) **Mice.** Female BALB/c and C57BL/6 mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany). Animals were  
25 to 8 weeks old at the onset of experiments and were kept under conventional conditions.

(B) **Parasites and preparation of antigen.** Parasites of the *Leishmania major* isolate MHOM/IL/81/FE/BNI (Solbach et al., Infect. Immun. 54: 909 (1986) were maintained by passage in BALB/c mice and were grown in  
30 conventional blood agar plates in vitro. For the preparation of total *L. major* lysate, stationary-phase promastigotes were collected, washed three times, resuspended at  $1 \times 10^9$ /ml in PBS and subjected to three cycles of freezing and thawing.

(C) **Oligonucleotides.** The oligonucleotide 1668 (CpG ODN, 5' TCCAT-GACGTTTCCTGATGCT 3') and the control AT-rich oligonucleotide (non-CpG ODN, 5' ATTATTATTATTATTA TTAT 3') were synthesized by MWG  
35 (Ebersberg, Germany) and were not phosphorothioate-modified.

(D) **Preparation and culture of bone marrow-derived dendritic cells (BMDC):** Dendritic cells (DC) were generated from bone marrow progenitors using the protocol of Lutz et al., J. Immunol. Meth. 223: 77-92 (1999)



5 with minor modifications. Briefly, total bone marrow cells were obtained from femurs and tibiae after flushing with a syringe containing PBS. Cell suspension was washed and resuspended in culture medium (Click RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 10 mM Hepes buffer, 60 µg/ml penicillin, 20 µg/ml gentamycin, 10 17 mM NaHCO<sub>3</sub> and 0.05 mM 2-mercaptoethanol). At day zero,  $2 \times 10^6$  cells were seeded in bacteriological petri dishes in a total volume of 10 ml culture medium containing 200 U/ml recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotek/Tebu, Frankfurt, Germany). Additional 5 ml of culture medium containing 200 U/ml 15 GM-CSF were added at days 3 and 6. After 10 days of culture, non-adherent DC were collected, resuspended at  $1 \times 10^6$  cells/ml in fresh culture medium containing 200 U/ml GM-CSF and were incubated overnight (approx. 18 hr) with 30 µl of parasite lysate per ml of culture volume (approx. equivalent to 30 parasites per cell) for antigen pulsing. For some 20 experimental groups, concomitant treatment with recognized inducers of BMDC maturation (lipopolysaccharide, LPS: 1 µg/ml, Sigma, Heidelberg, Germany); CpG and non-CpG ODNs: 25 µg/ml; anti CD40 mAb: 5 µg/ml, Pharmingen, Hamburg; and tumor necrosis factor alpha, TNF-α: 500 U/ml, Peprotec/Tebu, Frankfurt, Germany) was included during pulsing. Control 25 groups with only CpG, non-CpG ODNs and LPS were also included. After overnight incubation, the cells were washed to remove soluble parasite antigen and maturation inducers, and resuspended in PBS for further use.

**(E) Treatment of mice:** After antigen pulsing/maturation, BMDC were washed and resuspended in PBS, and  $5 \times 10^5$  cells were injected intrave- 30 nously (i.v.) into the tail vein of naive mice. Control mice were injected with PBS. One week later, mice were infected subcutaneously (s.c.) with  $2 \times 10^5$  (BALB/c mice) or  $2 \times 10^6$  (C57BL/6 mice) stationary-phase *L. major* promastigotes into the right hind footpad. The course of infection was monitored weekly by measuring the increase in footpad size, compared 35 with the uninfected contralateral footpad. For re-infection experiments, mice were infected with  $5 \times 10^5$  parasites into the left hind footpad 9 weeks after the primary infection, which means 3 weeks after complete healing of primary infection. For therapeutic immunization, mice were initially infected and subsequently treated on days 7, 0 + 7, 7 + 14 or 14 + 21 post-infection by i.v. injection with  $5 \times 10^5$  BMDCs.

- 5       **(F) Determination of the parasite load.** In order to analyze whether effective leishmanicidal mechanisms were taking place at the site of infection, the amount of viable parasites in the footpads was determined by a limiting dilution technique. Briefly, after 5-6 weeks post infection the right foot was removed, washed with ethanol and rinsed three times with PBS.
- 10       Preparation of soft tissues was performed by making some slits with a sterile scalpel and by macerating the foot in a cell strainer. Cell suspensions were then passed through a 30G needle in order to assure the release of intracellular parasites. Subsequently, suspensions were centrifuged for 5 minutes at 100g in order to separate tissue clumps and debris.
- 15       Serial dilutions of the supernatant in 100  $\mu$ l/well were seeded into 96-well microculture blood-agar plates. For each dilution, replicates of 20 wells were set up. After 10 days of incubation at 28°C in a humidified atmosphere with 5% CO<sub>2</sub>, the cultures were scored for the presence of parasites using an inverted microscope. The estimation of the number of parasites
- 20       per footpad was done by multiplying the reciprocal of the last dilution showing at least one positive well with the initial dilution factor. For some experimental groups, 10  $\mu$ l of the footpad cell suspension were smeared onto a glass slide, stained with Giemsa and observed in a conventional light microscope for the presence of *L. major* amastigotes.
- 25       **(G) Measurement of cytokine production.** Lymph nodes draining the infected footpads were removed 5 weeks after infection. After preparation of single-cell suspensions,  $1 \times 10^6$  cells were cultured in 1 ml volume (24-well plates) in the absence or presence of 10  $\mu$ l of parasite lysate for 72 hours. Thereafter, culture supernatants were harvested for the determination of
- 30       the cytokines IL-2, IL-4 and IFN-gamma by sandwich ELISA, as published previously (Flohé et al., Eur. J. Immunol. 28:3800-3811 (1998)). IL-12p70 was also measured by sandwich ELISA in supernatants of BMDC cultures after 20 hours of pulsing/maturation.
- 35       **(H) Determination of *Leishmania*-specific IgG antibodies.** Mice of the experiment shown in Fig. 1B were sacrificed 5 weeks after infection and *Leishmania*-specific IgG, IgG1 and IgG2a serum levels were assayed by ELISA. Plates were coated with total lysate (equivalent to  $5 \times 10^5$  parasites/well) and incubated overnight with mouse serum (dilutions: 1:100 for total IgG; 1:50 for IgG1 and IgG2a). For total IgG a second antibody (anti-mouse IgG-alkaline phosphatase conjugate) was incubated for 1 hour and

5 developed with a chromogenic phosphatase substrate. For IgG1 and IgG2a, 1-hour incubation with an isotype-specific second antibody (biotinylated rabbit anti-mouse IgG1 and IgG2a, respectively), 1 hour with streptavidin-conjugated alkaline phosphatase and final substrate development were used. Relative levels of antibodies are presented in optical density  
10 (O.D.).

(I) **RT-PCR.** Total RNA was isolated from BMDC cultures after 36 hours of different pulsing/maturation treatments, using the RNeasy total RNA extraction kit (Qiagen, Hilden, Germany) and 2 µg of RNA were reverse transcribed (Qiagen, Hilden, Germany). Primers for IL-12 p35, IL-12 p40 and β-actin (MWG Biotech, Ebersberg, Germany) were used in a PCR reaction  
15 to estimate the relative amount of their respective mRNAs.

20 **Example 2: CpG-matured / lysate-pulsed BMDC protect BALB/c mice from cutaneous leishmaniasis**

Recently, it has been reported that Langerhans cells that had been pulsed with *Leishmania* antigen confer protection against murine leishmaniasis. Initial attempts to reproduce this protective effect with a different population of DC, the BMDC, were unsuccessful. Several modifications of the  
25 protocol with regard to the time of BMDC generation, the amount of BMDC injected into the mice and different conditions of antigen pulsing were performed. However, no protection against infection could be observed (not shown). Thus, additional maturation stimuli of BMDC seemed to be re-  
30 quired. Therefore, a series of experiments was performed in which cells were not only pulsed with parasite lysate (as the source of antigen), but in addition treated with inducers of BMDC maturation, including LPS, anti-CD40 antibodies, CpG ODN and TNF-alpha. Two independent and representative experiments are shown in Fig 1. Again, antigen-pulsed BMDC  
35 were not able to induce protection against leishmaniasis (Fig 1B). When antigen-pulsed BMDC were additionally treated with the maturation inducers LPS, anti-CD40 and TNF-alpha, or combinations of those stimuli, BMDC were also unable to protect against leishmaniasis (Fig 1A and 1B). In contrast, immunization of mice with antigen-pulsed BMDC that had been cultured in the presence of CpG ODN conferred complete protection

5 against subsequent infection with *L. major* in otherwise susceptible  
BALB/c mice (Fig. 1A and 1B). All mice that had been vaccinated with  
those cells developed only a minor footpad swelling (always less than 1  
mm, Fig. 1A and 1B), which peaked 3 weeks after infection, and were  
10 completely cured after 6 weeks (Fig. 1A). None of the mice in this group  
showed any sign of ulceration. The course of lesion development in control  
groups immunized with non-pulsed CpG-treated BMDC or pulsed BMDC  
treated with a non-CpG motif AT-rich ODN was comparable to the PBS  
control group (Fig 1B). These findings demonstrate that a single i.v. injection  
15 with antigen-pulsed CpG-matured BMDC mediates complete protection  
against murine leishmaniasis.

### **Example 3: Clinical cure correlates with a significant reduction in parasite burden**

20 It was analyzed whether the protection induced by CpG-matured antigen-  
pulsed BMDC is paralleled by an effective control of parasite replication at  
the site of infection. Fig. 2A shows the parasite loads in individually ana-  
lyzed mice from the protected and the control groups. All mice that had  
25 been vaccinated with CpG/antigen-BMDC had a significantly lower para-  
site burden than the control mice. On average, there was a more than  $10^4$   
fold reduction in the number of parasites per footpad ( $7.3 \times 10^{11}$  and  $1.2 \times$   
 $10^7$  for control and protected groups, respectively). When smears from the  
control footpads were analyzed under the microscope, an uncountable  
30 high amount of parasites was seen and, as shown in Fig 2B, macrophages  
were typically full of intracellular parasites, indicating active replication. In  
contrast, in samples obtained from the protected footpads, parasites could  
hardly be detected and the typical observation was the presence of no or  
very few intracellular amastigotes (Fig 2B). These results indicate that the  
35 protection induced by immunization with CpG-matured antigen-pulsed  
BMDC in susceptible mice is due to an acquired ability to efficiently activate  
anti-*Leishmania* effector mechanisms.

5       **Example 4: CpG-matured / lysate-pulsed BMDC induce a shift in the cytokine profile**

10       In order to determine whether the protection induced by BMDC is associated with a different profile in cytokine expression, the secretion of IL-2, IFN-gamma and IL-4 by lymph node cells was assessed. Mice from the most relevant experimental groups shown in Fig. 1B were sacrificed 5 weeks after infection, and total lymph node cells were cultured for cytokine analysis by ELISA. The levels of IL-2 in the absence of *Leishmania* antigen ranged between 7.6 and 20.7 ng/ml with the maximal level exhibited by the protected group that had been vaccinated with BMDC-lysate-CpG. However, this difference was strikingly enhanced when *Leishmania* antigen was added to the culture. A 13-fold higher level of this cytokine was observed in the protected compared with the control and 2 to 4-fold higher than the other groups (Fig. 3A). An even more pronounced difference was observed when IFN-gamma levels were determined. As shown in Fig. 3B, a 10-fold increase was observed in the absence of antigen when the protected group is compared with the control group and 2 to 7-fold when compared with other groups. When *Leishmania* antigen was present in the cultures, a 151 and 16 to 60-fold higher level of IFN-gamma was observed when protected group is compared with control and the other groups respectively (Fig. 3B). In contrast to IL-2 and IFN-gamma, lymph node cells from mice belonging to the protected BMDC-Lysate-CpG group secreted no detectable, or very low, levels of IL-4 in the absence or presence of antigen, respectively (Fig 3C). Some of the non-protected groups were also low IL-4 producers. Thus, in mice treated with CpG-matured / lysate-pulsed BMDC, the cytokine profile induced in lymph node cells was strongly shifted towards Th1-like immune response.

35       **Example 5: The pattern of Leishmania-specific IgG antibodies correlates with the induction of a Th1 immune response in CpG-matured/lysate-pulsed BMDC- vaccinated mice**

It is well known that different IgG subclass profiles correlate with Th1 or Th2 immune response. The presence of high levels of IgG1 and low titers

5 of IgG2a anti-*Leishmania* antibodies is associated with a Th2 response and the reverse distribution with a Th1 response. Thus, it was investigated whether the pattern of IgG subclass production was shifted towards the Th1-type response in the protected group. Mice from the most relevant experimental groups showed in Fig 1B were sacrificed 5 weeks post infection, and the relative levels of total IgG, IgG1 and IgG2a antibodies were  
10 determined by ELISA. As shown in Fig. 4A, the levels of *Leishmania*-specific total IgG antibodies were variable but significant in all experimental groups. When the IgG subclass distribution was determined, a clear tendency to produce low IgG1 and high IgG2a levels was observed in the serum of protected mice that had been treated with BMDC-lysate-CpG (Fig. 4B and 4C). Some groups showed low levels of IgG1 and some high levels of IgG2a, but only the protective CpG matured/lysate-pulsed BMDC were able to induce the combination of both. A simpler parameter to see Th1-like shifting seems to be the relative ratio of IgG2a to IgG1, with  
15 higher values indicating Th1 induction. As showed in Fig. 4D, the protected BMDC-Lysate-CpG group exhibited the highest IgG2a/IgG1 average ratio which was 4 times higher than for the control group (1.4992 and 0.3661, respectively). Some other groups showed higher ratio values than the control group due to higher IgG2a levels, but in contrast to the protected group, they also exhibited higher levels in IgG1 than the control group. Taken together, these results indicate that only the protected group of mice, which was vaccinated with CpG-maturated lysate-pulsed BMDC, produces a pattern of anti-*Leishmania* antibodies that correlates with the induction of a strong Th1 immune response after infection with virulent *L.*  
20 *major*.  
25  
30

**Example 6: The protective effect of CpG-matured/lysate-pulsed BMDC is also observed in the resistant strain of C57BL/6 mice**

35

It was investigated whether this approach is also applicable to a different strain of mice which is resistant to *L. major* infection. As very well known (and shown in Fig 5A, control group), C57BL/6 mice develop a limited inflammation in the footpad after infection and finally cure after 6-8 weeks of infection. However, when these mice are vaccinated with CpG-

5 matured/lysate-pulsed BMDC one week before the infection, a significant  
reduction in the footpad swelling is observed, with a lower maximal peak  
and faster healing (Fig. 5A). When these mice are vaccinated with BMDC  
alone, an initial unspecific effect is observed. However, these mice  
reached a footpad swelling comparable to the control after 4-5 weeks post-  
10 infection (Fig 5A). As expected, vaccination with BMDC treated with CpG  
alone showed no effect. In contrast to BALB/c mice, C57BL/6 mice vac-  
cinated with BMDC pulsed with lysate in the absence of CpG treatment also  
showed a reduction in lesion development, when compared with non-  
vaccinated mice, but this effect was less pronounced than that induced by  
15 antigen-pulsed BMDC further matured by CpG ODN treatment (Fig. 5A).  
When the parasite load of the different vaccination groups was analyzed, a  
striking correlation with the clinical outcome was observed. The parasite  
numbers of mice with non-protective treatment were similar to those of the  
control (Fig 5B). Mice vaccinated with lysate-pulsed BMDC showed a 10-  
20 fold reduction in the parasite load. Most notably, those mice vaccinated  
with CpG-matured lysate-pulsed BMDC had approximately 100-fold less  
parasites in the footpads (Fig. 5B). These results demonstrate that vac-  
cination with CpG-matured lysate-pulsed BMDC induces a significant pro-  
tective effect, with a reduction of the parasite load at the site of infection, in  
25 both BALB/c and C57BL/6 mice.

**Example 7: Resistance induced by CpG-matured / lysate-pulsed  
BMDC immunization is solid and protects against re-infection**

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Because of the exceptional efficacy of CpG-matured/lysate-pulsed BMDC  
in mediating protection (total cure of 100% of the mice, Fig. 1), it was in-  
vestigated whether the mice that resolved the primary infection were able  
to resist a second challenge with parasites. To this end, the 10 mice that  
35 completely cured in the experiment shown in Fig. 1A were rechallenged  
with  $0.5 \times 10^6$  metacyclic parasites (2.5-fold more than the primary infec-  
tious dose) 10 weeks after the first challenge. The results in Fig. 6A show  
that solid immunity was established by immunization with these BMDC,  
since the swellings developed after secondary infection was even lower  
than those after primary challenge. Rechallenged mice showed an almost

5 unreadable footpad swelling (less than 0.5 mm) and most of them completely cured after 3 weeks after the secondary infection. This group of mice was followed up for more than 20 weeks after secondary challenge without any sign of disease.

10 **Example 8: The immunotherapeutic effect of CpG-matured/lysate-pulsed BMDC is dependent on the time of BMDC administration**

Given the unusual potency of these cells in inducing a long-lasting protective Th1 immune response, the next question to address was whether it is possible to cure an already established *Leishmania* infection. For this purpose, a series of experiments was designed in which naive mice were infected and subsequently treated with CpG-matured/lysate-pulsed BMDC at different time points. The results shown in Fig. 6B indicate that under our experimental conditions, in a very limited time window of not longer than 7 days after infection, it is still possible to redirect the immune response towards a protective phenotype. When mice are treated on day 0 (1 hour post infection) and one week after infection, a very clear therapeutic effect is observed. When therapy is performed one and two weeks after infection the effect is reverted, since not only no curative effect is observed but also the treatment seems to be exacerbating. A similar curve of disease progression is observed when a single therapeutic dose is injected one week after infection, indicating that the therapeutic efficacy exerted by the schedule 0+7 days p.i. is more dependent on the first dose than the second, and strongly challenges the use of this approach at least in this model and under our experimental conditions. Finally, two therapeutic doses on days 14 and 21 p.i. did not show any effect as evidenced by a kinetic in disease progression comparable to that from the control group (Fig. 6B). Thus, a therapeutic application of BMDC in murine leishmaniasis is possible. However, the time of administration seems to be critical for efficacy.

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**Example 9: CpG-matured / lysate-pulsed BMDC express IL-12**

10 To explore the mechanisms involved in the activation of the protective Th1-like immune response observed in mice vaccinated with CpG-matured/lysate pulsed BMDC, the expression of IL-12 was analyzed. This cytokine is formed by the subunits p40 and p35 and is known to play a key role in the development of Th1 cells. For this purpose, BMDC were treated as described in Example 1, and after 36 hours total RNA was isolated and levels of IL-12 p35 and p40 mRNAs were determined by RT-PCR. Super-

15 natants of the same cultures were also collected and the active p70 form of the protein was measured by ELISA. As shown in Fig. 7A, p40 and p35 mRNA were differentially regulated by pulsing/maturation stimuli in BMDC. CpG and non-CpG ODN as well as LPS induce a very strong up-regulation of p35 mRNA while parasite antigen pulsing down-regulates both basal

20 and induced expression. Among the groups having been treated by pulsing and maturation stimuli, BMDC-CpG combination showed the maximal p35 mRNA level. In contrast to p35, basal levels of the p40 mRNA were apparently unchanged by pulsing only. However, except for lysate-CpG combination, pulsing down-regulated the inducible expression (see LPS and CpGco alone versus LPS-Lys and CpGco-Lys, respectively). Again,

25 among groups having been treated by pulsing and maturation stimuli, BMDC-CpG combination showed the maximal p40 mRNA level. Active p70 protein levels in supernatants were also dependent on the pulsing/maturation treatment as shown in Fig. 7B. As expected, maximal levels of p70 subunit were induced by CpG ODN treatment of BMDC. No p70 IL-12 was detectable in non-treated, pulsed only, and CD40 or TNF-alpha-matured pulsed cultures. However, in spite that again pulsing down-regulated the LPS- and CpG-induced p70 production, once more the last

30 one was the treatment exhibiting the maximal level of functionally active p70 IL-12 among pulsed/matured groups (at least doubling amounts). All these results suggest that the protective effect observed when susceptible mice are vaccinated with BMDC pulsed with *Leishmania* lysate and matured with CpG ODN, is due to the induction of a strong Th1 immune response, which is correlated with the ability of these cells to secrete active

35 p70 IL-12.

5

**Claims**

- 10 1. A not naturally occurring dendritic cell (DC) having specific antigen presentation properties in an individual comprising a specific disease related antigen and a CpG molecule, wherein said DC derives from CD34<sup>+</sup> bone marrow precursor cells or peripheral blood monocyte preparations.
- 15 2. A DC according to claim 1, wherein the CpG oligonucleotide comprises the nucleic acid sequence  
5'-TTCATGACGTTCTGATGCT-3'
- 20 3. A DC according to claims 1-2, wherein said DC was obtained by prolonged exposure to IL-4 and GM-CSF in vitro.
4. A DC according to claims 1-3, wherein the antigen is a microbial or a cancer antigen.
- 25 5. A DC according to claims 4, wherein the microbial antigen derives from a parasite.
- 30 6. A DC according to claims 5, wherein the parasite is Leishmania.
7. A DC according to claims 4, wherein the specific antigen presentation property comprises a Th1 type immune stimulatory response.
- 35 8. A pharmaceutical composition comprising a DC of any of the claims 1-7, optionally together with a pharmaceutically acceptable carrier, diluent and excipient.

- 5        9.     A pharmaceutical composition according to claims 8 comprising a  
         cytokine.
10.    A vaccine comprising a DC according to claims 1-7 and an addi-  
         tional adjuvant.
- 10       11.    Use of DC according to claims 1-7, for the preparation of a me-  
         dicament for the prophylactic or therapeutic treatment of infectious  
         and cancerous diseases.
- 15       12.    A method of producing a DC, comprising the following steps:  
         (a) exposing a specific antigen to the isolated DC as defined to any  
         of the claims 1-7, and  
         (b) treating the DC with one or more CpG oligonucleotides
- 20       13.    The method of claim 12, wherein the DC derives from mobilized  
         stem cells and is isolated from peripheral blood, wherein said pre-  
         cursor cells have been cultured under conditions allowing to gener-  
         ate functional DCs.
- 25       14.    The method of claim 12, wherein step (a) and (b) are carried out  
         simultaneously.
- 30       15.    A pharmaceutical kit comprising several packages, wherein a first  
         package contains DC obtained from CD34<sup>+</sup> bone marrow precursor  
         cells or peripheral blood monocyte preparations by culturing the  
         cells in IL-4 and GM-CSF, a second package with a CpG molecule  
35       and a third package with a disease related antigen.

**Fig. 1**

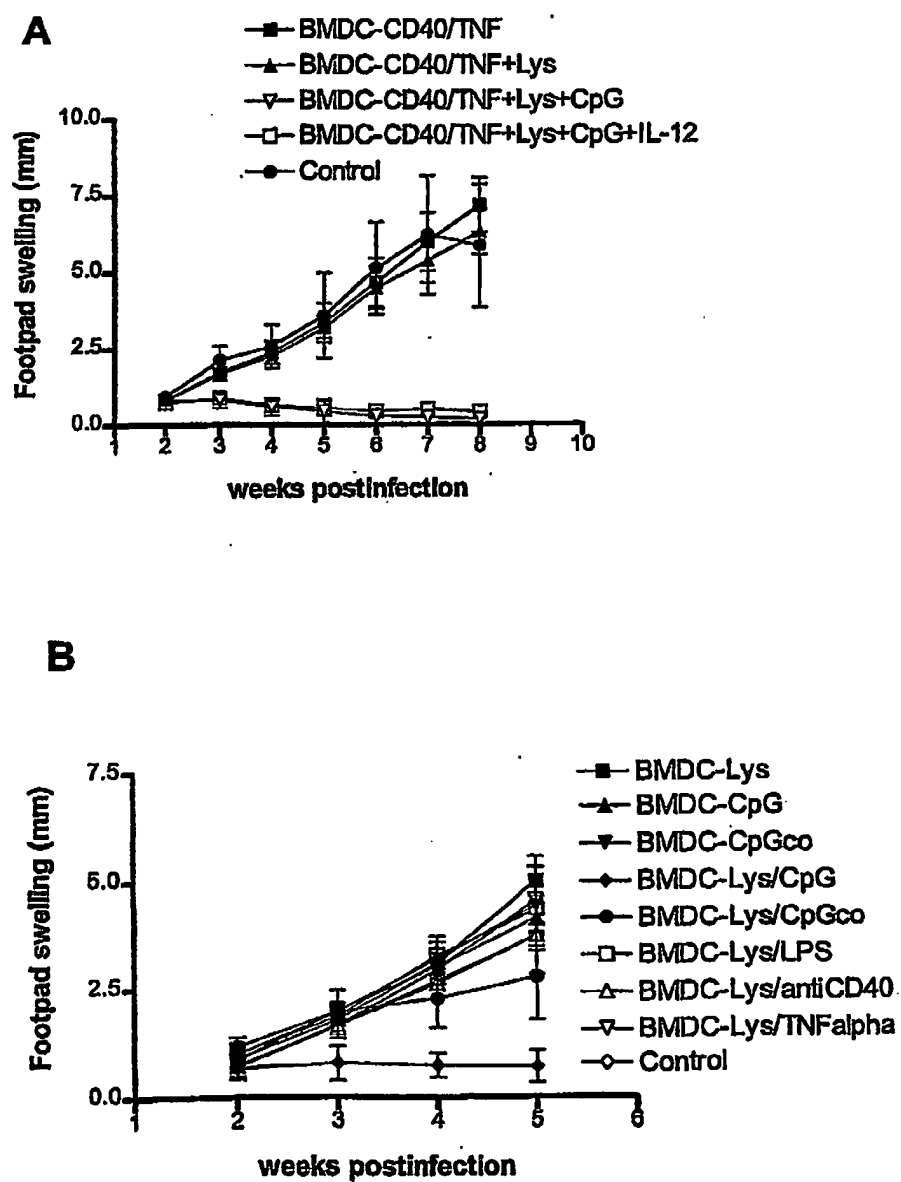
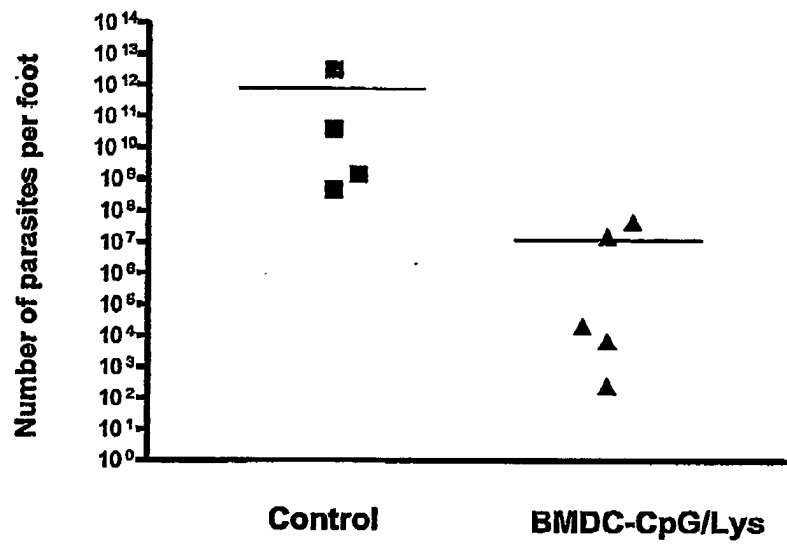


Fig. 2

A



B

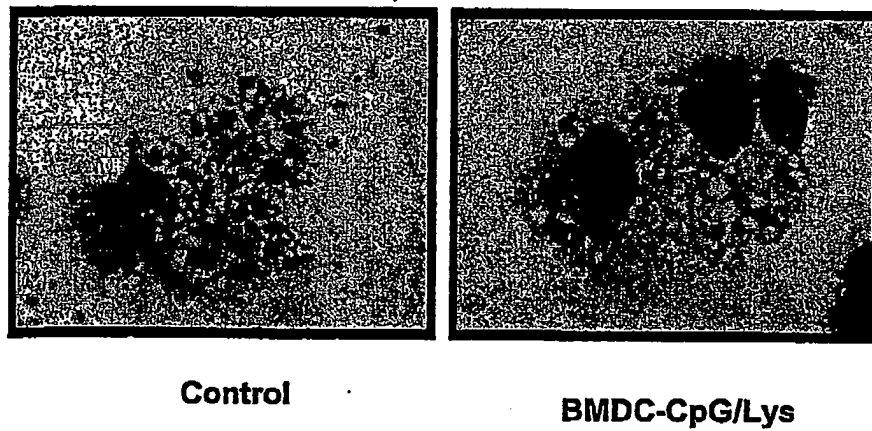
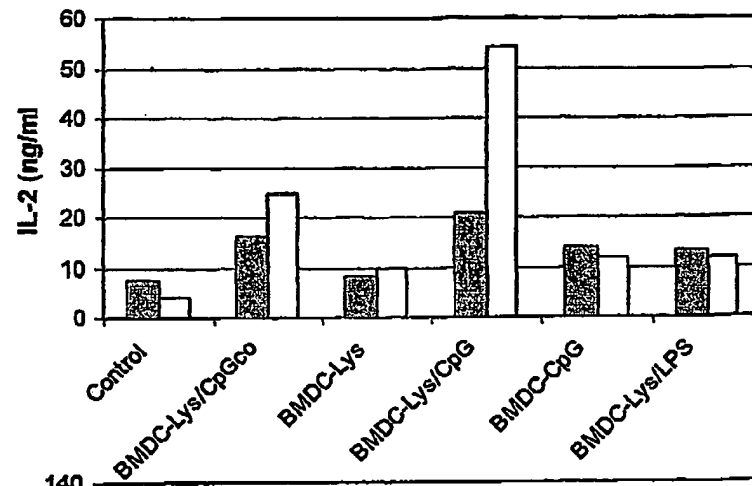
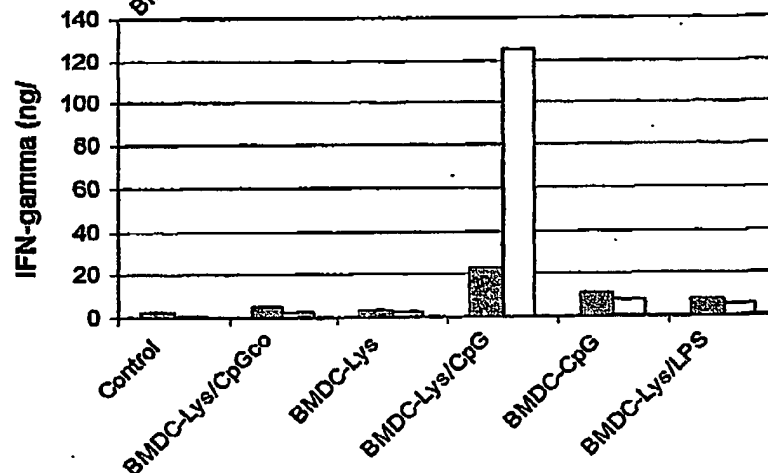


Fig.3

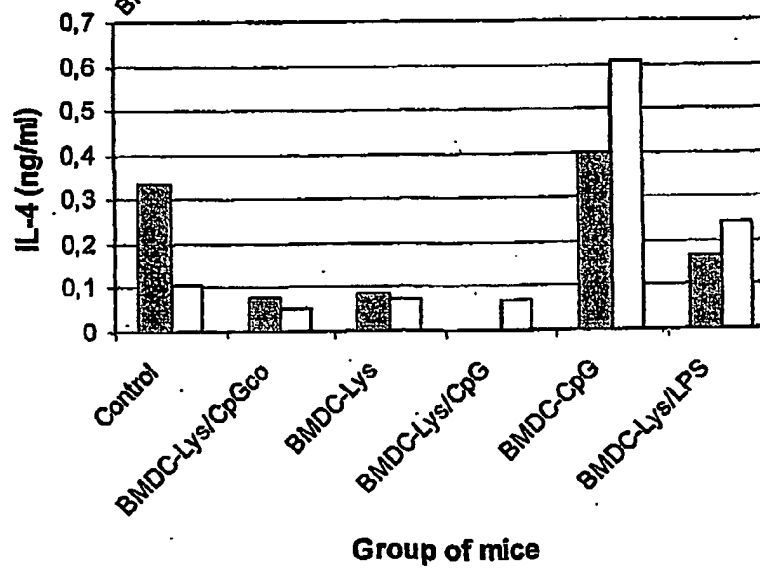
A



B



C



■ No Ag  
□ Ag

Fig. 4

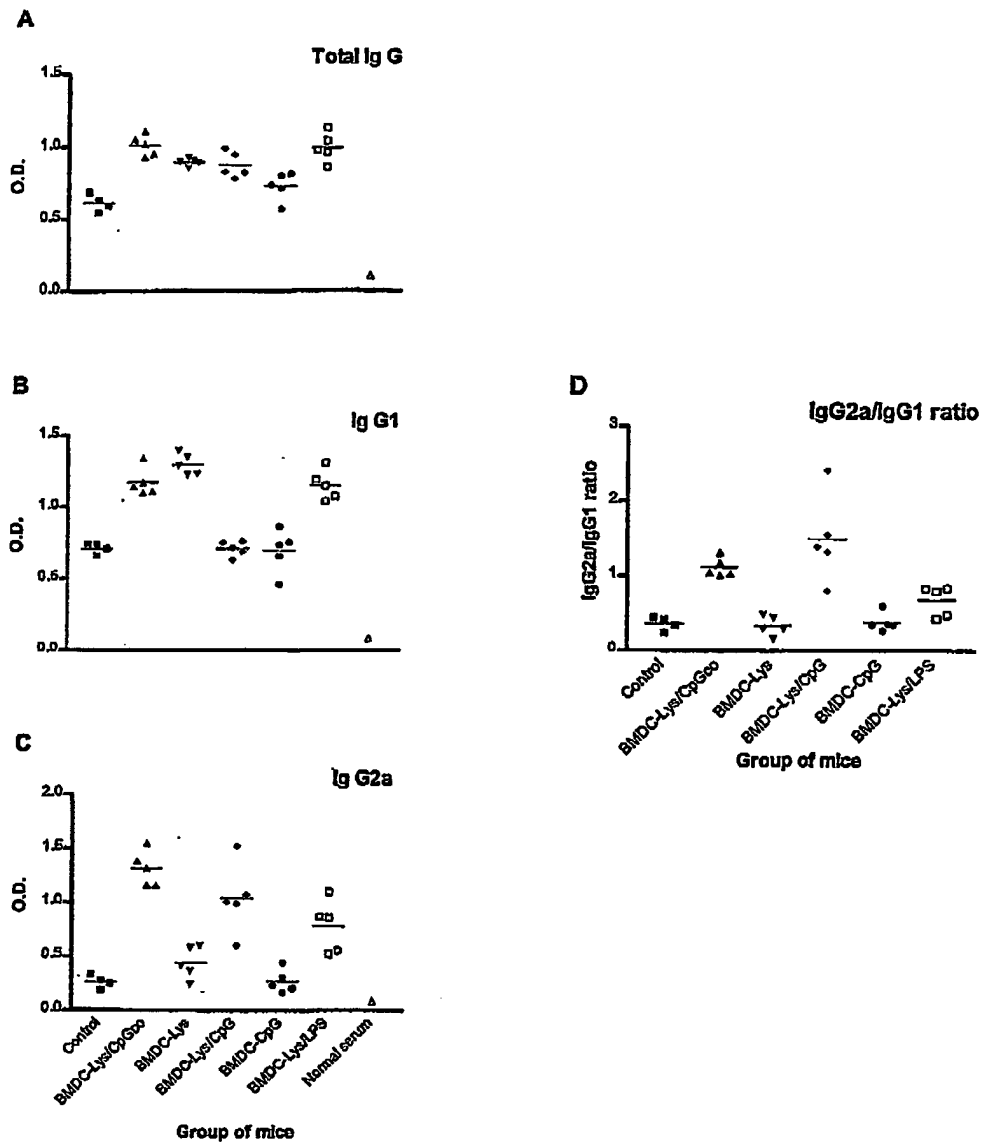


Fig. 5

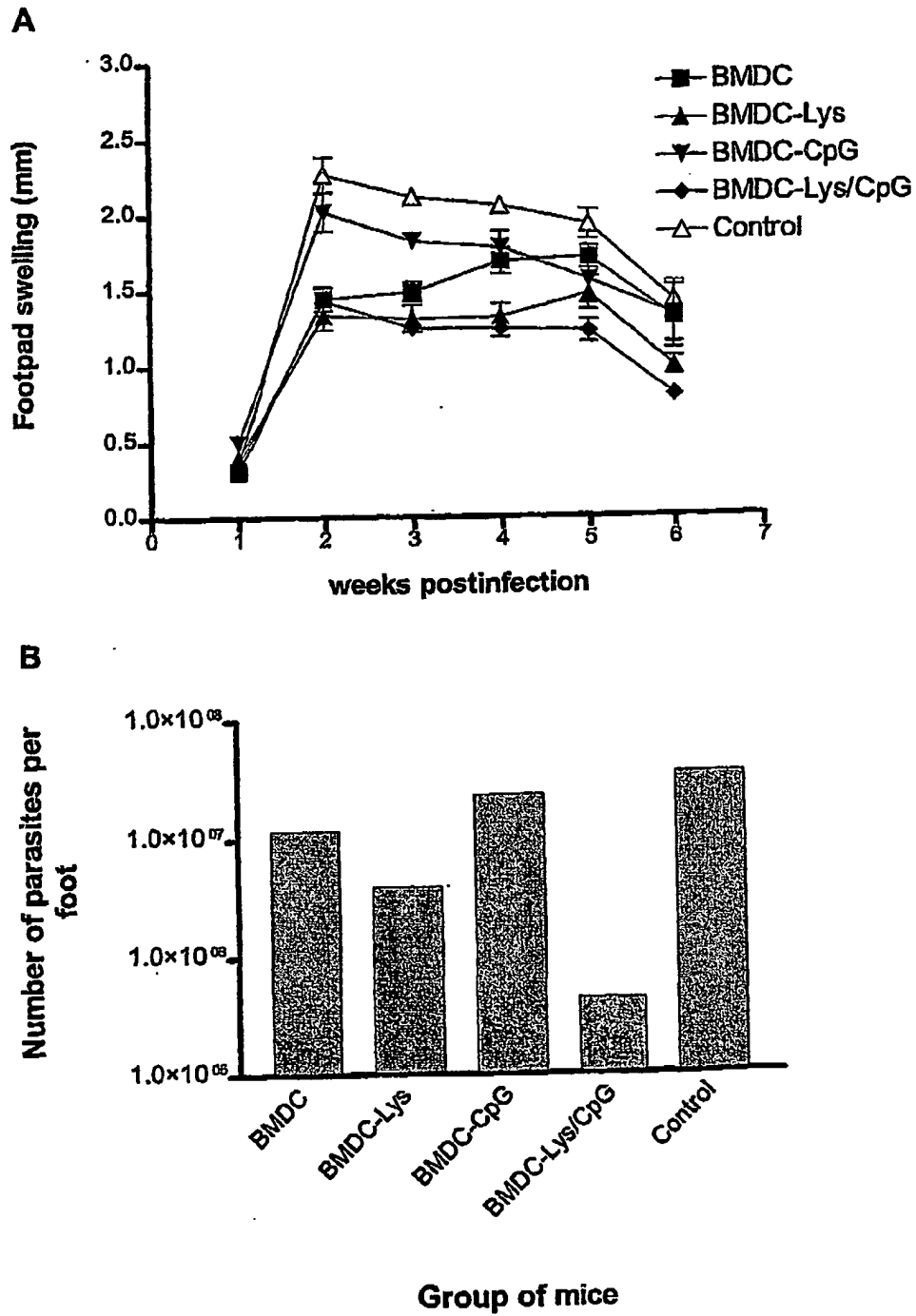
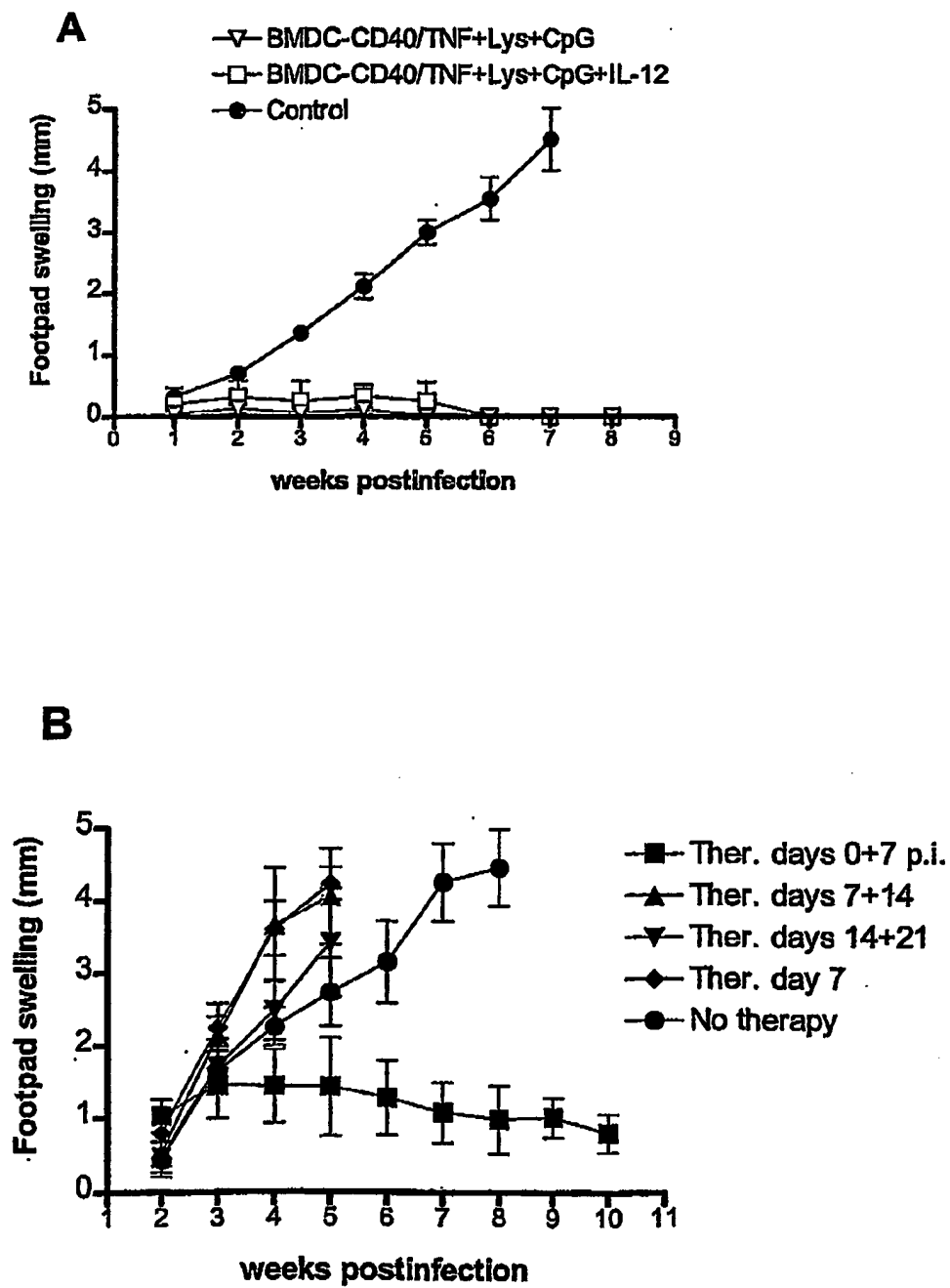
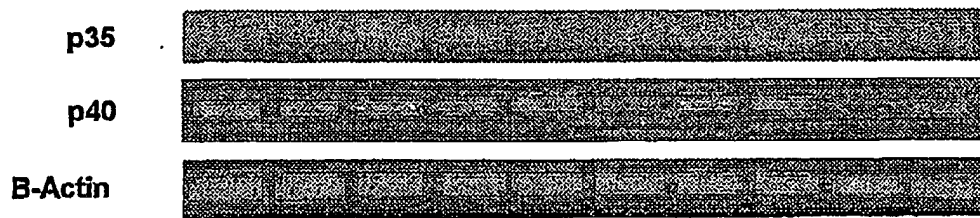
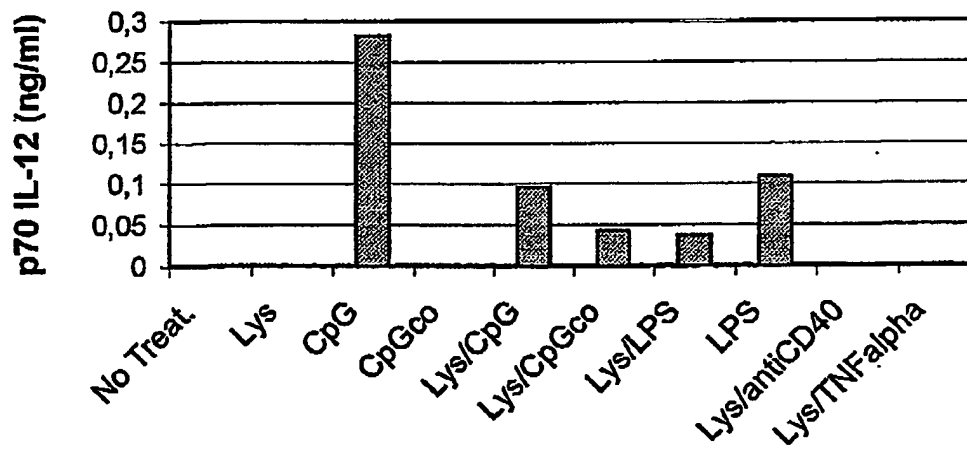




Fig. 6



**Fig. 7****A****B**

## INTERNATIONAL SEARCH REPORT

Inter Application No  
PCT/EP 03/05567A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12N5/06 A61K39/00 A61K39/008

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, BIOSIS, MEDLINE, EMBASE, SEQUENCE SEARCH, PAJ, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WAGNER H: "BACTERIAL CPG DNA ACTIVATES IMMUNE CELLS TO SIGNAL INFECTIOUS DANGER" ADVANCES IN IMMUNOLOGY, ACADEMIC PRESS INC., NEW YORK, NY, US, vol. 73, 1999, pages 329-368, XP009015569 ISSN: 0065-2776 the whole document, in particular paragraph VI, p. 336-338 — —/—	1-15



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

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Date of the actual completion of the international search

28 August 2003

Date of mailing of the international search report

17/09/2003

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## INTERNATIONAL SEARCH REPORT

Info Application No  
PCT/EP 03/05567

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JAKOB T ET AL: "ACTIVATION OF CUTANEOUS DENDRITIC CELLS BY CpG-CONTAINING OLIGODEOXYNUCLEOTIDES: A ROLE FOR DENDRITIC CELLS IN THE AUGMENTATION OF TH1 RESPONSES BY IMMUNOSTIMULATORY DNA" JOURNAL OF IMMUNOLOGY, THE WILLIAMS AND WILKINS CO. BALTIMORE, US, 1998, pages 3042-3049, XP002928254 ISSN: 0022-1767 the whole document	1-15
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